Duplication of chicken *defensin7* gene generated by gene conversion and homologous recombination

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Defensins constitute an evolutionary conserved family of cationic antimicrobial peptides that play a key role in host innate immune responses to infection. Defensin genes generally reside in complex genomic regions that are prone to structural variation, and defensin genes exhibit extensive copy number variation in humans and in other species. Copy number variation of defensin genes was examined in inbred lines of Leghorn and Fayoumi chickens, and a duplication of *defensin7* was discovered in the Fayoumi breed. Analysis of junction sequences confirmed the occurrence of a simple tandem duplication of *defensin7* with sequence identity at the junction, suggesting nonallelic homologous recombination between *defensin7* and *defensin6*. The duplication event generated two chimeric promoters that are best explained by gene conversion followed by homologous recombination. Expression of *defensin7* was not elevated in animals with two genes despite both genes being transcribed in the tissues examined. Computational prediction of promoter regions revealed the presence of several putative transcription factor binding sites generated by the duplication event. These data provide insight into the evolution and possible function of large gene families and specifically, the defensins.

antimicrobial peptides | copy number variation | defensin | nonallelic homologous recombination | chicken

Genetic variation provides the basis for natural selection and genome evolution. Whereas studies of genetic variation have historically focused on single nucleotide polymorphisms (SNPs), structural changes are now known to be common in animal genomes. Copy number variants (CNVs), duplications or deletions that segregate in breeding populations, have gained considerable interest as a major source of genetic variation. CNVs are important to phenotypic diversity and evolutionary adaptation in animals and plants, using a variety of mechanisms, such as gene dosage and transcript structure alterations to modulate organismal plasticity (1). Considerable advances have been made in the characterization of the genomic architecture of CNVs in animal species. In recent years, advances in high-throughput genome scan technologies, particularly DNA hybridization on array platforms and next-generation sequencing methods, have allowed the identification of a large number of structural variants in multiple species. Although not as comprehensive as those for human, CNV maps have been produced in cattle (2, 3), horse (4), sheep (5, 6), pig (7), goat (8), duck (9), and chicken (10, 11).

CNVs can be generated by at least three general mechanisms, homologous recombination (HR), nonhomologous recombination, and retrotransposition (12, 13). HR requires extensive DNA sequence identity of approximately 50 bp in *Escherichia coli* (14), and as many as 300 bp in mammals (15, 16) with breakpoint regions surrounded by repetitive sequences. Nonallelic homologous recombination (NAHR) on the same chromosome between low copy and segmental duplications is a common mechanism to generate CNVs. Nonhomologous recombination does not require homology and needs only short microhomologies (several bp) for repair. Nonhomologous end joining (NHEJ) and fork stalling and template switching (FoSTeS) are alternative models. FoSTeS produce microhomology, whereas NHEJ can produce loss or gain of several nucleotides at the joint (17). Retrotransposition, usually involving long interspersed elements such as L1, does not require homology and generates nonspecific insertional variation (18).

Defensins are a family of short cationic peptides involved in host defense, immunomodulation and reproduction. The defensins are broadly divided into five groups, namely plant, invertebrate, and the α-, β-, and θ-defensins found in vertebrates, including mammals. Evolutionary studies suggest that the formation of defensin gene families resulted from ancient gene duplication events. For example, all α-defensin genes are thought to have evolved by gene duplication after the bird-mammal split and θ-defensins appear to have arisen from α-defensins (19, 20). Humans have α- and β-defensin (*BD*) genes that map to 8p23.1 and vary independently in copy number, although the exact size and breakpoints of the variable segments are not known (21). Accurate measurement of multiallelic copy number variants is challenging, particularly for high copy numbers, and has not been intensively addressed until recently (22). Structural variation in genes underlying host immunity can affect disease susceptibility. CNV of human defensins are associated with susceptibility to Crohn's disease, psoriasis, and some cancers (23, 24). However, whereas CNVs of defensins in the human genome have received considerable attention, intraspecific CNVs in other animal species are poorly studied.

The chicken genome encodes a total of 14 *BD*s with no other group of defensins known, and they are clustered densely within approximately 90 kb on chromosome 3 (20, 25). To better understand the role of *BDs* in the immune system and to investigate mechanisms of CNV generation in animal genomes, we examined CNV in the *BD* gene family. Here, we report copy number variation of *BD7* in highly inbred Leghorn and Fayoumi lines and

Significance

Copy number variation is an important source of genetic variation in animal genomes and an evolutionary intermediate in gene family expansion. In this study, we report the presence of a tandem duplication of β-defensin *7* (*BD7*) among multiple breeds of chickens and a resulting chimeric promoter that appears to result from gene conversion followed by nonallelic homologous recombination. These findings contribute to the understanding of gene family expansion in animals and the role of large gene families in host-defense mechanisms.

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propose that duplication of *BD7* arose by gene conversion followed by homologous recombination.

Results

Identification of a *BD7* Duplication in Fayoumi Chicken. We used whole genome sequencing to reveal structural variation in Brahma, Fayoumi, and Hamburg chickens. Whole genome sequencing with 10× coverage revealed a duplication at about GGA3:107,901,000 bp–107,905,000 bp in Fayoumi compared with the other breeds. This observation suggested an increased copy number in the *BD7* region of Fayoumi (Fig. 1). DNA from inbred Leghorn Ghs6 and Fayoumi M5.1 lines was then used to explore CNV of the *BD7* gene. Quantitative real-time PCR (RT-qPCR) analysis with PF2 and PR2 primers (Fig. 2*A*) revealed that these inbred Fayoumi chickens had a two-fold increase in copy number of *BD7* relative to inbred Leghorn Ghs6 individuals (Fig. 2*B*).

Junction Sequence Reveals a Direct Tandem Duplication. PCR was used to demonstrate the presence of a direct tandem duplication of the *BD7* region. PCR primers, PF3 and PR1 (outward facing relative to reference sequence; Fig. 2*A*) located at the predicted boundaries of the duplicated regions, were used to amplify the duplication junction. PCR amplification with primers PF2 and PR2 that were previously used to detect CNV was used as an internal control, and amplicons were detected in all examined birds, indicating that they all have at least one copy of *BD7*. The junction sequence was detected in Fayoumi but not in Leghorn chickens, confirming a duplication of *BD7* in the former breed (Fig. 2*C*). The sequence of the junction region showed that the duplicated segments are joined in the same orientation, and the duplication of *BD7* occurred between GGA3:107,900,788 bp and GGA3:107,904,653 bp within homologous sequences with shared break point end sequences (Fig. 3*B*). These data confirmed that the entire *BD7* gene is tandemly duplicated.

Duplication Event Generated Two *BD7* Copies with Sequence Variation of Promoter. We amplified and sequenced the *BD7* gene and found a variant in the promoter region. In detail, the primer pairs Inter F1 and R2.1 (Fig. 2*A*) were used to amplify and sequence animals with a single copy of *BD7* located near *BD8*, which we call *BD7a*. Unexpectedly, when we sequenced *BD7* in animals with two *BD7* copies, neither copy had the promoters identified in *BD7a*. Further investigation of this region demonstrated a sequence we designated *BD7b*, containing an interrupted sequence of approximately 150 bp with a replacement of the *BD7a* promoter sequence with sequence from the equivalent region of the *BD6* promoter. We propose this arrangement to be a result of gene conversion (Fig. 3*A*). Interestingly, the *BD6* and *BD7a* promoters share a 38-bp sequence that is 100% identical and could have promoted the gene conversion event.

The second copy in the duplicated region of *BD7* contains 590 bp upstream of the start codon and 2,618 bp downstream of the stop codon and this 5′ flanking sequence harbors approximately 160 bp of the *BD6* promoter region. This 160 bp of *BD6* promoter sequence was added upstream of the *BD7* promoter by nonallelic homologous recombination, resulting in a chimeric promoter between *BD6* and *BD7*. We designate this gene as *BD7c* (Fig. 3*B*).

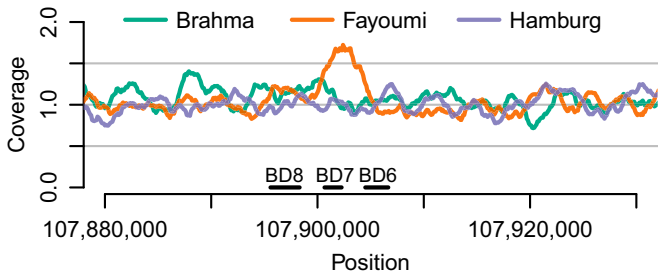

Fig. 1. Whole-genome sequencing alignment indicating duplication of the *BD7* region in Fayoumi chicken. The relative coverage is shown for the target region on chromosome 3. The coverage was calculated as mean coverage for a 2,001-bp sliding window. Each breed is represented by pooled DNA from five individuals.

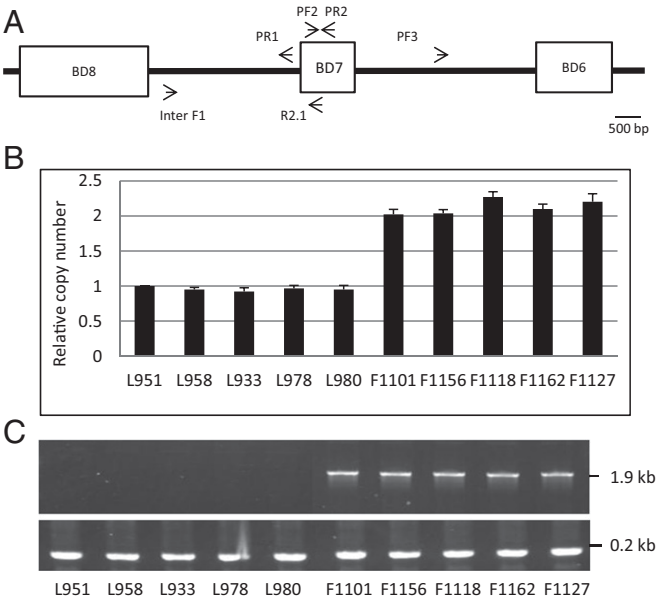

Fig. 2. A twofold increase of *BD7* copy number in an inbred Fayoumi line. (*A*) Schematic diagram of primers used. (*B*) Genomic copy number was estimated by using qPCR for one individual of each of five inbred lines of Fayoumi relative to Leghorn lines with PF2 and PR2 primers. Fayoumis show an estimated copy number of ~2 per haplotype assuming Leghorns carry single-copy haplotypes. The relative abundance of *BD7* was assessed by normalization and calibrated to *PCCA*. F, Fayoumi; L, Leghorn. Each bar represents the mean ± SD value of three independent experiments. (*C*) Duplication junction identified by PCR in Fayoumi lines. The junction of the duplicated region amplified only in Fayoumi with outward-facing primers, PF3 and PR1. Internal control was amplification of *BD7* across all birds with primers, PF2 and PR2.

Duplication of *BD7* generated two chimeric sequences of its promoter, *BD7b* and *BD7c* (Fig. 3). We also examined SNP frequency in coding versus noncoding regions of *BD7*. The two *BD7* genes (*BD7b* and *BD7c*) share exactly the same sequences in their cDNA, except for two SNPs in 5′ UTR that are generated by the duplication (Fig. 4 *B* and *C*). Only one SNP was detected in the first intron between *BD7b* and *BD7c*, but none in other intron or exon regions.

Distribution of the *BD7* Duplication in Chicken Breeds. To understand the evolutionary relationship of duplicated *BD7*, we tested copy number in red junglefowl, considered to be the wild ancestors of domesticated chickens. All six red junglefowl individuals tested had a single copy of *BD7*. Sequence analysis revealed that one animal was heterozygous for *BD7*, exhibiting both the *BD7a* and *BD7b* single copy sequences. Thus, we learned that *BD7b* also occurs in a single copy haplotype, and not exclusively in duplication with *BD7c*. We designated alleles at the *BD7* locus and their associated sequences as follows: *7A* (*BD7a*), *7B* (*BD7b*), and *7C* (*BD7b* and *BD7c*). A PCR screening revealed that the *BD7* duplication was present in Hamburg, Buttercup, and Silkie animals, although we did not find it in Plymouth Rock and White Leghorn (Table 1). RT-qPCR revealed that two Hamburg animals showed 1.5-fold changes compared with Leghorn. Further sequencing analysis revealed that these animals were heterozygous *7A*/*7C*.

Expression of *BD7*. We performed RT-qPCR to examine the effects of the CNV on *BD7* expression in bone marrow tissue in which *BD7* genes are highly expressed. The results indicated no significant difference

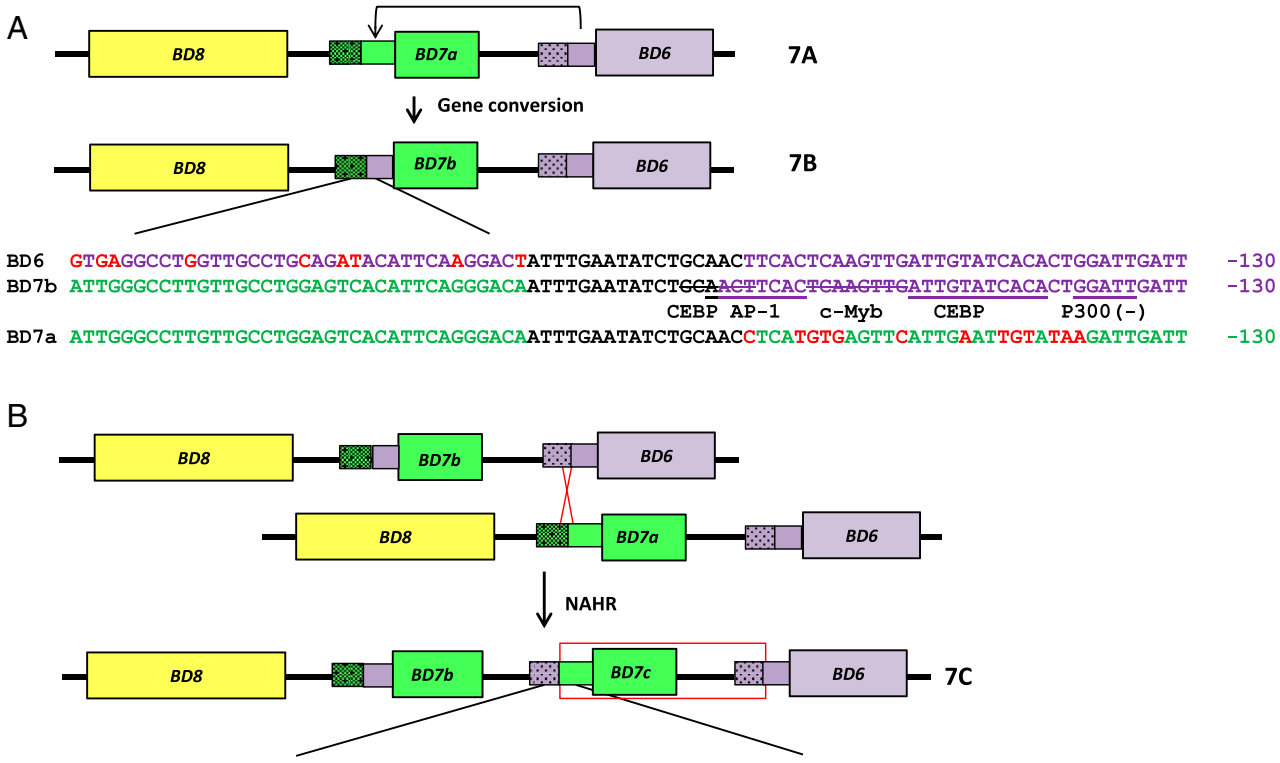

Fig. 3. Schematic overview of predicted order and orientation of duplicated *BD7* genomic structure. (*A*) Gene-conversion events occurring between homologous sequences of *BD6* and *BD7a* that reside on same chromatids generate *BD7b*. Gene-conversion events that occur between homologous sequences that reside on sister chromatid are not shown. Sequence alignment of the *BD6* and *BD7* promoters are shown below. The proximal sequence from *BD7a* and distal sequence from *BD6* are shown in green and purple, respectively, and variant nucleotides are marked in red. The number in the right indicates distance from the start codon. Putative transcription factor binding sites are underlined. AP-1, activator protein-1; CEBP, CCAAT/enhancer binding protein. (*B*) NAHR upstream of *BD6* and *BD7a* resulted in a duplicated *BD7* with a chimeric promoter, producing *BD7c*. Duplicated region is boxed in red. Sequence alignment of duplication junction points is shown below. The proximal sequence from *BD6* and distal sequence from *BD7a* are shown in purple and green, respectively, and variant nucleotides are marked in red. CREM, cAMP-response element modulator.

between genotypes in expression levels of *BD7* (Fig. 4*A*). *BD7b* and *BD7c* mRNA sequences are identical except for their 5′ UTR that was generated by the duplication and gene conversion event. It prompted us to test for expression of both *BD7* alleles by amplifying 5′ UTR through 3′ UTR. Sequencing data revealed that Fayoumi birds produce two transcripts, *BD7b* and *BD7c*, whereas Leghorns produce only *BD7a* (Fig. 4*B*).

Prediction of Putative Transcription Factor Binding Sites. The genomic region upstream of the *BD7* was searched for the presence of transcription factor binding sites associated with *BD7* regulation. Computational prediction of this region revealed the presence of several putative transcription factor binding site in BD7b and BD7c promoter regions including activator protein-1 (AP-1), c-Myb, CCAAT/Enhancer Binding Protein α and β (CEBPα and CEBPβ), p300, and cAMP-response element modulator (CREM tau) binding protein site (Fig. 3). These binding sites were generated by the duplication event, and the CEBP binding site, in particular, was newly created by combining BD6 and BD7 promoters by gene conversion.

Discussion

We have demonstrated that the duplication of *BD7* involves the fusion of the 5′ and 3′ end of the *BD6* and *BD7*, respectively, by tandem duplication. Tandem duplication is one of the major gene duplication mechanisms in eukaryotes, as illustrated by the prevalence of gene family clusters. Because *BD7* gene duplicates are so close to the original parental gene, we have no reason to believe that transposition participated in this duplication. Absence of additional sequence and high inside sequence homology in the break point region present the possibility of recombination. We found sequence identity at the center of the breakpoint junction of duplicated regions with high similarity between two sequences. So we can cautiously rule out the possibility of NHEJ and FoSTeS. We propose that the arrangement occurred by

Table 1. Presence of the *BD7* duplication in different breeds of chicken

Population	Presence of duplication	Absence of duplication	Total	Presumed genotype
Hamburg	2	2	4	2:7A/7C 2:7A/7A
Buttercup	1	3	4	1:7C/7C 3:7A/7A
Silkie	2	2	4	2:7C/7C 2:7A/7A
Plymouth Rock	0	4	4	4:7A/7A
White Leghorn	0	4	4	4:7A/7A
Red junglefowl	0	6	6	5:7A/7A 1:7A/7B
Leghorn Ghs6	0	8	8	8:7A/7A
Fayoumi M5.1	8	0	8	8:7C/7C

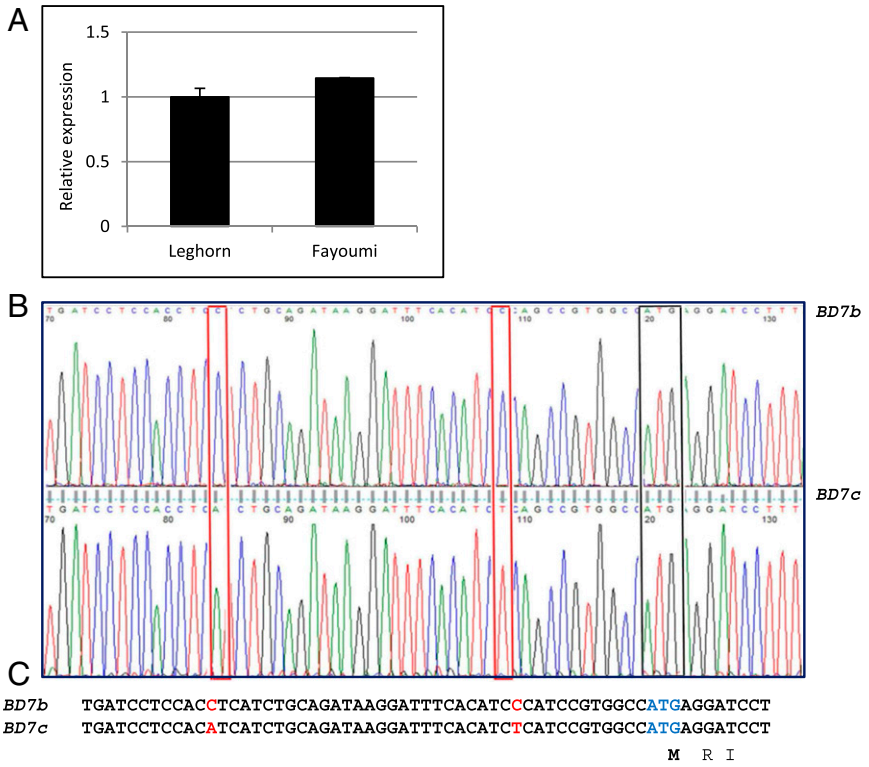

Fig. 4. Comparison of spatial expression pattern of defensin7 genes. (*A*) qRT-PCR analysis *BD7* gene expression in bone marrow. The relative abundance of mRNA was assessed by normalization and calibrated to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). Each bar represents the mean ± SD value of three independent experiments. ***$P \leq 0.001$, **$P \leq 0.01$, and *$P \leq 0.05$. (*B*) Sequencing chromatography of 5′ UTR of *BD7b* and *BD7c*. Two SNPs and start codon are marked in red and black boxes, respectively. (*C*) Sequence of *BD7b* and *BD7c*. Two SNPs and start codon are marked in red and bold, respectively.

NAHR, and this event generated chimeric promoters of *BD7c* by adding 150 bp of the *BD6* promoter to the sequence upstream of the promoter of *BD7* (Fig. 3).

The *BD7b* sequence present in the *7B* and *7C* allele has an interruption of 150-bp sequences, a replacement of the *BD7* promoter sequence with the sequence from the corresponding region of the *BD6* promoter, from an apparent gene conversion event. *BD6* is considered to have originated by a duplication of *BD7* after the *Gallus gallus-Poephila guttata* split (19). The two genes share high sequence similarity in their promoter and first exonic region but are quite different after the first intron in both their lengths and sequences. The high sequence similarity between *BD6* and *BD7* with a possible double-stand break (DSB) in the promoter of *BD6* may have led to the direct transfer of the *BD6* fragment into the promoter of *BD7*, resulting in *BD7b*. In general, the rate of gene conversion is negatively correlated with the tract distance in mammals (26), and gene-conversion tracts are usually short, of the order of 200 bp to 1 kb in length (27). Duplicate copies of *BD7* and *BD6* are separated by approximately 7 kb with high sequence identity and a predicted gene conversion tract length up to 350 bp. Gene conversion most likely explains the difference between the *BD7a* and *BD7b* sequences. The Gallus_gallus-5.0 assembly of the chicken genome revealed a single *BD7a* copy that was confirmed by our analysis of a male and a female bird of the red junglefowl inbred line UCD001, the line from which the reference bird originated. One of six red junglefowl individuals examined, however, was heterozygous for a single copy haplotype with the *BD7b* sequence, suggesting that *BD7b* existed before the duplication event. Individuals with the duplication always have a copy of *BD7b* and *BD7c* at this locus, suggesting that duplication resulted in the unique additional *BD7c* copy by NAHR in an animal heterozygous for *BD7a* and *BD7b*. These findings support the hypothesis that the duplicated genomic region of *BD7* was generated by two steps, gene conversion to produce *BD7b*, followed by homologous recombination in a heterozygous animal (Fig. 3).

Duplicates of *BD7* with chimeric promoter sequences prompted us to examine its expression pattern in various tissues. RT-qPCR analysis revealed that the duplication was not associated with increased expression in bone marrow, which is known to show high expression levels of defensin genes. We also detected similar expression levels of *BD7* in thymus and liver in chickens with single and duplicated genes (Fig. S1). Our data are consistent with the observation that most young tandem duplicates demonstrate coregulated gene expression to the level of the single copy gene and are thereby permitted to evolve slowly (28). *BD7* expression in Fayoumi chicken undergoes dosage sharing, which may allow long-term survival of the duplicated copy. As with other examples of dosage sharing, we found that both *BD7b* and *BD7c* were expressed in all tested tissues, although Sanger sequencing of transcripts suggested a higher expression of *BD7c* than *BD7b* in Fayoumi (Fig. S2). Successful duplication requires the maintenance or acquisition of gene regulatory modules. We analyzed promoter sequences of *BD7* to examine transcription factor binding sites. c-Myb binding sequences along with other transcription factors, such as CEBPα, CEBPβ, and P300/CBP (CREB binding protein), known to be involved in transcriptional transactivation of target promoters with c-Myb, are found in the *BD7b* promoter (29). Binding sites for the transcription factor AP-1 that regulates gene expression in response to a variety of stimuli, including cytokines, growth factors, stress, and bacterial and viral infection was found in *BD7b*, generated by the gene conversion event (30). CREM mediates signal transduction during the spermatogenetic cycle and is also involved in the genetic component conferring general susceptibility to inflammatory bowel disease. These findings suggest that duplication of *BD7* does not enhance

induced gene expression but may be involved in differential expression after stimulation or involved in male fertility. More investigations on the role of the duplication of *BD7* in developmental and spatial regulation after stimulation are necessary. CNV can impact transcriptomes on a global scale by modifying the expression of genes that localize within the CNV and on its flanking sequence, an effect that can extend over hundreds of kilobases from the breakpoints (31). Because defensin genes play an important role in host innate immune responses to infection, further analysis of other immune response genes and flanking gene expression is justified.

Defensin gene families map to dynamic regions of genomes in different animal groups (21), and CNV is often associated with segmental duplications (18) or other unstable elements. The chicken BD cluster covers the sequence from coordinates 107,930,407–107,850,580 on chromosome 3. Using the avian Evolution Highway website (http://eh-demo.ncsa.uiuc.edu/birds/#/SynBlocks), we found these coordinates to be totally or partially represented in an evolutionary breakpoint region (EBR) in 12 of the 20 avian aligned by Farré et al. (32). This site therefore plays a potential role in chromosomal evolution and in gene family dynamics.

Materials and Methods

Experimental Animals. The Fayoumi samples used for whole-genome sequence alignment belong to the AvianDiv collection and originate from a line kept by Institut National de la Recherche Agronomique (INRA) in its Pôle d'Expérimentation Avicole de Tours facility, France. The Brahma samples originated from the flock of a French fancy breeder, Jacques Berger, Villereau, France. The Hamburg samples originated from the same flock of Jacques Berger (three animals) and from the flock (two animals) of another French fancy breeder, Frédéric Theme, Saint Jeanvrin, France. White Leghorn, Plymouth Rock, Silkie, Buttercup, and Hamburg breeds came from Ideal Poultry Breeding Farms, Inc. (Cameron,TX) and were maintained in veterinary medical research park at Texas A&M University under conditions of the approved Institutional Animal Care and Use Committee protocol 2014-0315.

Chicks of the highly inbred Leghorn Ghs-6 line and the Fayoumi M5 line were produced and maintained in the Poultry Genetics Program at Iowa State University. Birds were raised in light- and temperature-controlled pens with wood-shaving bedding and continual access to water and food, meeting all National Research Council (NRC) nutritional requirements. At 7 wk of age, birds were euthanized according to the approved Institutional Animal Care and Use Committee protocol (log no. 4-03-5425-G) and tissues were immediately dissected and were placed into RNAlater until used for isolation of mRNA.

Whole Genome Alignment. Three pooled samples, each representing DNA from five individuals of the Brahma, Fayoumi, or Hamburg breeds, were prepared and sequenced to ~10× coverage by using Illumina HiSeq with 2 × 100-bp paired-end reads. After removal of adapter sequences, the reads were aligned to the chicken reference genome (galGal5) by using bwa aln (33) with the parameters "–t8 –q20". Read coverage was calculated by using samtools mpileup (34) and normalized by the sample mean.

Detection of Copy Number Variation by Quantitative PCR. Whole blood was collected from the wing vein of each bird of Fayoumi and Leghorn lines into EDTA-coated anticoagulant tubes. Genomic DNA was extracted from blood with DNeasy Blood and Tissue Kit. DNA quality was assessed by spectrophotometer (NanoDrop 1000), and 50 ng was used as a template. Quantitative PCRs (qPCRs) were performed by using LightCycler 480 SYBR Green master mix (Roche) following the manufacturer's instruction. All qPCRs were performed in triplicate with Roche 480 lightcycler and technical replicates of $n \geq 3$ were used. Data were analyzed by the ddCt method to normalize the target Ct value to the reference Ct value within samples and, subsequently, to normalize all samples to a known calibrator sample. The calibrator sample in copy number variation analysis was one of the Ghs6 Leghorn individuals, L951. A propionyl CoA carboxylase (*PCCA*) single copy gene was used as standard. Primer sequences are listed in Table S1.

Junction Sequence PCR Amplification Using Outward Facing Primers. Assuming tandem direct duplication and using outward facing primers, PCR was performed by using high-fidelity polymerase (GXL polymerase, TAKARA) to amplify junction regions. The reaction conditions were the following: 98 °C 1 min; followed by 98 °C for 10 s, 68 °C for 5 cycles, 67 °C for 5 cycles, and 65 °C for 25 cycles for 10 s, and 68 °C for 3 min; followed by 68 °C for 1 min. Amplification products of PF3 and PR1 were recovered by electrophoresis, and DNA was analyzed by sequencing.

RNA Isolation and qPCR. Spleen, thymus, duodenal loop, liver, bone marrow, and bursa were collected from 7-wk-old chicks for RNA isolation. Total RNA was isolated from each tissue of chickens of the Leghorn line (Ghs6) and Fayoumi lines (M5.1) by using the RNeasy kit, and cDNA was obtained by reverse transcriptase SuperScript III First-Strand Synthesis System using total RNA as a template. The relative abundance of mRNA from genes was assessed by real time reverse-transcription–PCR using a Lightcycler 480 (Bio-Rad) and a Lightcycler 480 SYBR Green I master (Bio-Rad). PCR products were subjected to melt curve analysis and sequenced to confirm amplification of the correct gene. Data were analyzed by the ddCt method to normalize the target Ct value to the reference Ct value within samples and subsequently normalized to the known calibration sample. Each analysis was performed in triplicate. Quantification of each sample was calculated with the cycle threshold values and standard curve information by using the Lightcycler 480 version 1.5.0 software.

Promoter Prediction. To examine the regulatory mechanism of defensin 7 expression, we investigated the presence of putative transcription factor binding sites for approximately 740 bp of 5′ upstream region of defensin7 by using the ALLGGEN-PROMO (alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) multisearch site.

Statistical Analysis. Data were expressed as mean ± SD of three independent experiments. Statistical analysis was performed by using the Student's *t* test. Differences were considered to be statistically significant at $P < 0.05$.

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